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Reproductive isolation between *Stigmaeopsis celarius* and its sibling species sympatrically inhabiting bamboo (*Pleioblastus* spp.) plants

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Abstract *Stigmaeopsis celarius* Banks (hereafter *Sc*) is a spider mite living and feeding on the leaves of various bamboo species such as Moso bamboo [*Phyllostachys edulis* (=*P. pubescens*)] and *Pleioblastus* spp. (Poaceae). A previous phylogenetic study revealed a cryptic, phylogenetic sister species to *Sc* (hereafter *Ss*). Although its life type appears to be similar to that of *Sc*, individuals of *Ss* make much smaller nests compared with *Sc*, and the nests have been found mostly on Nezasa bamboo (*Pleioblastus argenteostriatus*). To investigate whether *Sc* and *Ss* are reproductively isolated, we explored their populations in southwestern Japan, and crossed them to examine mating behaviors and fertilization success. Field surveys revealed that the nests of these two species occur on the same leaves and, thus, the individuals of these species may make frequent contact. Reciprocal crosses suggested that the two species are reproductively isolated. Though *Sc* males have tried to mate with *Ss* females, copulation seldom occurred because of their long opisthosoma (hind body), which prevented the insertion of the aedeagus into the genitalia of *Ss* females. In contrast, most *Ss* males ignored *Sc* females, and eggs were not fertilized even in the few cases where copulation appeared to occur. These results suggest that strong selection pressure is imposed on body length to prevent interspecific hybridization in the contact area of these species.

Keywords Cryptic species · Mechanical isolation · Reinforcement · Reproductive interference · Secondary contact zone · Tetranychidae

Introduction

In many organisms, multiple species that differentiated from a common ancestor can be redistributed in the same geographic area, forming a secondary contact zone. These species can mate with other species and even form hybrids (Futuyma 1998; Coyne and Orr 2004). This is a form of reproductive interference, i.e., interspecific interaction that adversely

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affects the fitness of at least one species involved in mate acquisition (Gröning and Hochkirch 2008). One ecological consequence of the reproductive interference is that one species can be excluded from the contact zone through reproductive interference without resource allocation or niche partitioning (Bull 1991; Kuno 1992; Takafuji et al. 1996; Kishi et al. 2009; Kyogoku and Nishida 2013). Another consequence is that various kinds of mating traits change in the contact zone. In the reinforcement theory, selection against hybridization favors the evolution of premating isolating mechanisms (Gröning and Hochkirch 2008). Behavioral, life history, and morphological traits would be evolutionarily changed to prevent hybridization because hybrids usually suffer from lower fitness than their parental species (Gröning and Hochkirch 2008). Clarifying the process of mating interaction between proximately redistributed species is important to realize selection pressure on behavioral and morphological traits of each species, and to infer the possibility of sympatric coexistence of these species.

Stigmaeopsis spider mites (Acari: Tetranychidae) feed on bamboo species, e.g. *Sasa* spp. and *Pleioblastus* spp., or Chinese silver grass *Miscanthus sinensis* (Poaceae). They are suitable materials to investigate the consequences of a secondary contact zone on reproductive characters, because different species live on the same host-plant shoots across a wide range in Japan, i.e., they are sympatrically or parapatrically distributed (Mori and Saito 2004; Sato et al. 2008). The life type of *Stigmaeopsis* species is characterized by woven nests (Saito 1985). On the undersurface of host leaves, individuals construct densely-woven silken nests along the midrib, in the depressions of leaves, or on the curly parts of leaf edges. Offspring grow up and reproduce inside the nest, and in species making large nests, offspring of the following generations often live together in the same nest. Colony members are engaged in enlarging and repairing nests as well as sanitary behavior in several cooperative social species (Saito 1983, 1997, 2010; Sato et al. 2003; Sato and Saito 2006).

Both precopulatory and postcopulatory reproductive isolations are reported in the *Miscanthus*-inhabitor, *Stigmaeopsis miscanthi* (Saitō 1990). Two genetically differentiated forms (HG and LW) are parapatrically distributed and form the boundary of the distribution area in the mountainous regions of Japan (Sato et al. 2008). Crossing experiments suggest that interform copulation is partly successful (as inferred from the increase in fecundity compared with virgin females), so that prezygotic reproductive isolation is incomplete (Sato et al. 2000b). In addition, the eggs fertilized by the other form did not develop fully, though a few developed into adult females in a particular combination of populations (Sato et al. 2000a, b, 2008). Thus, reproductive isolation occurs at both prezygotic and postzygotic stages in the two *S. miscanthi* forms (Sato et al. 2000b). Precopulatory isolation exists between certain pairs of bamboo-inhabiting species (Saitô and Takahashi 1982; Mori 2000). The nest made by mites functions as an obstacle against intrusion of different species. For example, *Stigmaeopsis longus* (Saitō 1990), who has a large body size, could not enter the nest of *Stigmaeopsis saharai* (Mori 2000), who makes the smallest nests in this group (Mori and Saito 2004). These studies suggest that the mechanisms of reproductive isolation among *Stigmaeopsis* species are diverse.

Based on the phylogenetic analysis of the cytochrome oxidase subunit I (*COI*) region, Ito and Fukuda (2009) discovered a cryptic strain (clade no. 4) closely related to *Stigmaeopsis celarius* (hereafter *Sc*) (clade no. 3). Although this cryptic strain has not been taxonomically described, we treat this strain as a species (hereafter *Ss*) in terms of the phylogenetic species concept (Coyne and Orr 2004), and regard the term *species* as a taxon corresponding to a monophyletic clade in Ito and Fukuda (2009). Note that this usage differs from Ito and Fukuda (2009), in which a single clade contained morphologically different variants, and each variant was assigned a species name according to the key provided by Saito et al. (2004) based on the morphological species concept (Coyne and Orr 2004).

Ss makes much smaller nests than *Sc* along major leaf veins or in the depression adjacent to leaf edges of *Pleioblastus* spp., but seldom inhabits the leaves of *Sasa* or *Phyllostachys* spp., on which other *Stigmaeopsis* species frequently live (K. Ito, personal observation). Occasionally, *Sc* and *Ss* live on the same *Pleioblastus* leaves. Thus, the opportunity for secondary contact of individuals of these species may exist in the wild, though the frequency of their encounters is unknown.

In this study, we first surveyed the parapatric and/or sympatric occurrence of the *Sc* and *Ss* species in the field to measure the frequency of their encounter. Second, we conducted crossing experiments to investigate whether they are reproductively isolated. In addition, we studied the mating behavior of males using video recording to seek for factors explaining the results. Finally, we investigated the lengths of idiosoma and opisthosoma (hind body) in males, which are closely linked with posture during copulation.

Materials and methods

Field survey of co-occurrence

We surveyed the field occurrence of *Sc* and *Ss* on *Pleioblastus* spp. in southwestern Japan from 2007 to 2014. The co-occurrence of two species was determined on a discrete size of web nests on the same or adjacent plants. Infested leaves were collected and kept in plastic bags in a cooler box. In the laboratory, adult mites were prepared into slide specimens using Hoyer's medium and observed under a phase-contrast microscope (BX50, Olympus, Tokyo, Japan) for identification purposes. When identification was difficult, the sequence of a partial *COI* region was used as supplementary information (Ito and Fukuda 2009; Ito et al. 2011).

The morphology of *Ss* closely resembles *Stigmaeopsis takahashii* (Saito et al. 2004) in that the length of dorsal seta d1 exceeds the base of the next posterior hair (e1), but does not reach to the base of the following hair (f1) (Saito et al. 2004; Ito and Fukuda 2009). However, the host plants and distributions of the two species differ: *S. takahashii* feeds mainly on *Sasa* spp. and is distributed in cooler regions such as the Hokkaido district. Furthermore, a phylogenetic study showed that "SC Tsukuba" and "SC Suita" specimens, which are *Ss* and *Sc*, respectively, in the present study, formed a single clade distinct from that of *S. takahashii* (Ito et al. 2011).

Host plants were morphologically identified according to Suzuki (1996) and Uchimura (2005). The scientific names followed the database index of the Botanical Gardens (YList [http://bean.bio.chiba-u.jp/bgplants/ylist_main.html]).

Mites

An experimental population of each species was collected from a field of the Yokonami peninsula, Tosa, Kochi, Japan (N33.429° E133.452°, WGS84). Though the contact zone of the two species was at this location, the experimental population of each species was collected 40–70 m away from the contact zone. Though the dispersal ranges of these species are unknown, microsatellite analyses in the two-spotted spider mite *Tetranychus urticae* showed a positive autocorrelation only between the subpopulations within 2.4–3.6 m for a rose greenhouse (Uesugi et al. 2009a) or 10–24 m for apple orchards

(Uesugi et al. 2009b), so that the gene flow of *T. urticae* by crawling or short-distance aerial dispersal may be limited to a short range. Assuming that *Sc* and *Ss* have similar dispersal ability as *T. urticae*, we considered that the present collection sites were sufficiently distant to hamper short-scale gene flow.

Rearing cultures were maintained on a detached leaflet of *P. argenteostriatus* resting on water-soaked cotton pads in a Petri dish (internal dimensions of 91.3 × 38.2 mm, Insect Breeding Dish; SPL Life Sciences, Gyeonggi-do, Korea) at 25 °C, 60 % RH (relative humidity) and LD (light:dark) 16:8 h conditions in a plant growth chamber (LPH-240S, Nippon Medical & Chemical Instruments, Osaka, Japan). All experiments were conducted under the same conditions.

Nest size

The nest size of each species was measured as follows. A detached *P. argenteostriatus* leaf was securely fixed on the interior angle of a polyvinyl chloride (PVC) angle (1 × 1 cm) using double-faced adhesive tape to provide the mites with a constant physical structure for nest weaving (Mori and Saito 2006). This angle was placed on the water-soaked cotton pads; the leaf surface was raised and wet cotton was packed under the angle to provide support. This method is analogous to Mori and Saito (2006, 2013) except for the angle materials (acryl and PVC) and host plant.

A young mated female (3–10 days after molting to adult) randomly collected from the rearing stock was individually released on the leaflet. Forty-eight hours after introduction of the female, nests were photographed with a scale using a digital video camera recorder (NEX-VG20H, Sony, Tokyo, Japan) attached to a stereomicroscope (SZX7, Olympus). Images were analyzed using ImageJ ver. 1.46r software (NIH, http://imagej.nih.gov/ij/), and the maximum lengths of the major and minor axes were measured for each nest. The nest size was calculated as the product of these lengths assuming a rectangular shape (Mori and Saito 2006, 2013). The average nest size was compared by the Welch *t* test (this method led to a decimal degree of freedom).

Cross experiment

A teleiochrysalis (quiescent deutonymph) female of one species arbitrarily collected from the rearing culture was allowed to make a nest on a 1 × 1-cm leaf square. After 2 days, one matured male of the same or another species randomly collected from each culture was introduced onto the leaf square. The success of male intrusion into the nests was checked every day, and the number of eggs produced was recorded on day 14. After removing parental mites, all offspring were reared to adulthood to determine hatchability, egg-to-adult survival, and sex ratio. The number of replicates for each cross ranged between 15 and 18.

A one-way analysis of variance (ANOVA) and Tukey multiple comparisons were used to compare each subject between crosses. Sex ratio data were arcsine-root transformed before analysis.

Copulation behavior

Copulation consists of several elements (Cone 1985). Upon finding a matured virgin female, an adult male first tries to mount the female body, then slips himself under the female abdomen from behind, holds her legs with his front legs, and bends his opisthosoma (posterior part of the body behind the transverse crease) upward to insert his extruded aedeagus into the female genital opening (Cone 1985; Oku 2014; Oku and Saito 2014). In this study, the raising of the opisthosoma was defined as “copulation behavior” because preliminary observations revealed that most males completed copulation after this step in intraspecific copulation.

A teleiochrysalis female randomly sampled from the culture of each species was transferred onto a small leaf square (3 × 3 mm). After 24 h, when the adult female emerged and made a nest, an adult male of the same or different species was introduced onto the piece of leaf, and copulatory behavior was recorded for 6 h using a digital video camera recorder (NEX-VG20H) attached to a stereomicroscope (SZX7). Fifteen replicates were collected from each cross, though a few were accidentally lost. Based on the video data, the following four points were measured for each pair: (i) the proportion of males showing copulation behavior; (ii) the number of copulation attempts (i.e., raising the opisthosoma upward); (iii) copulation time: the period of deep contact of the male opisthosoma with the female genital opening. If contact occurred many times per replicate, the average values were used for analyses; and (iv) the time from the first encounter of a male with a female to the onset of mounting behavior.

These data were compared pairwise among crossing treatments using the nonparametric Steel–Dwass test because their distributions were highly non-normal. The proportion of pairs in which copulation occurred was compared between the crosses using the chi-square test adjusted using Ryan’s procedure for multiple comparisons (Ryan 1960).

Body length

To measure body length, each individual used in the experiments was individually mounted on glass slides (No. 2215, Matsunami Glass, Osaka, Japan) with a droplet of Hoyer’s solution and covered with a cover glass. A 10-g weight was placed on the cover glass to flatten the mite body (Saito et al. 1999). The specimens were kept at 50 °C in an incubator (SIB-35, SANSYO, Tokyo, Japan) for 3 days. A 100× image was recorded using a microscope camera (DP20, Olympus) and analyzed using ImageJ ver. 1.46r (NIH).

The opisthosoma length (OL) of male mites was inferred from the distance between the bases of the dorsal setae C1 and PA (Fig. 2) (Lindquist 1985; Saito et al. 2004) because the

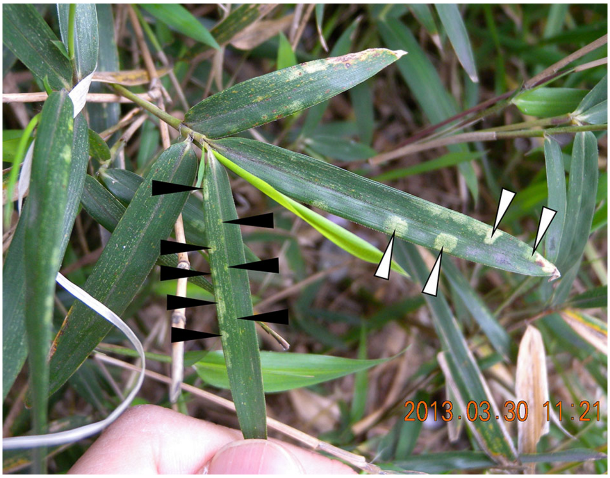

Fig. 1 Co-occurrence of *Stigmaeopsis celarius* (*Sc*) and *S.* sp. (*Ss*) on the same shoot of *Pleioblastus chino* in Kamakura, Japan. *White and black triangles* indicate the leaf scars made by *Sc* and *Ss*, respectively

mite body is soft and collapses easily. We measured only male OL because this is the length of the body part erected in copulation, and is closely related with the successful contact of aedeagus at the tip of male opisthosoma with female abdominal genital opening. We also estimated the total body (idiosoma) length [IL, the distance between the base of setae P1 (anteriormost hair of the prodosum) and PA] for each sex. Because C1, P1, and PA were approximately on the same straight line (Fig. 2b), the length of the prosoma (PL), i.e., the distance between P1 and C1, was estimated simply by subtracting OL from IL. The body lengths of each sex were compared between species using the *t* test. Prior to the analyses, an *F* test was conducted to test the homogeneity of variances. The Welch *t* test was applied if variance was unequal between groups.

To examine the effect of body size on copulation, we determined the correlation between female IL, male IL, the difference between them, and each copulation behavior (ii–iv) using Pearson's product moment correlation. Analyses were conducted on data from each intra- or interspecific cross (ii: $n = 6–14$, iii: $n = 6–13$, iv: $n = 3–10$) and on data pooled from all crosses.

Results

Field survey of co-occurrence

The *Sc* and *Ss* populations co-occurred at seven locations in Japan (Fig. 1; Table 1). Their host plants were limited to several species of *Pleioblastus* except for Moso bamboo at one site. The species were found on the same shoots in four areas and on adjacent shoots (at intervals ≤ 5 m) in five areas of Japan.

Nest size

The nest areas of *Sc* and *Ss* were 6.99 (mean) ± 2.55 (SD) mm^2 and 2.96 ± 1.02 mm^2 ($n = 10$, respectively). The difference between the nest sizes was highly significant ($t_{11.805} = 4.647$, $P < 0.001$, Welch *t* test).

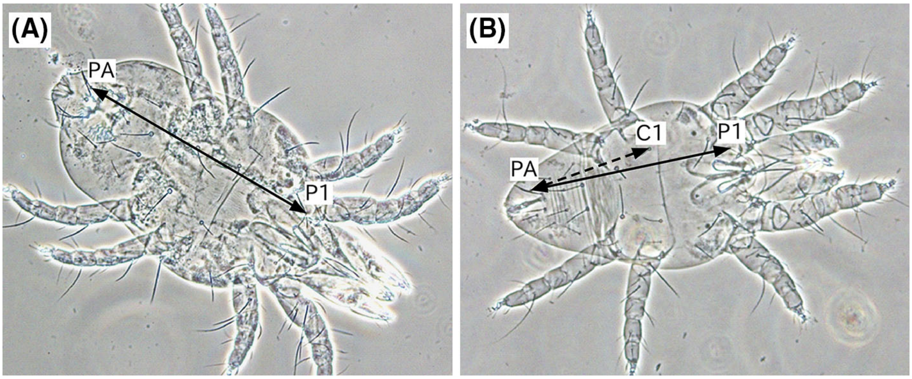

Fig. 2 Dorsal view of **a** female and **b** male *Stigmaeopsis* sp. Setae P1, C1 and PA are indicated. The bases of these three hairs in male are almost on a straight line, and the base of C1 is located near the crevice between prosoma and opisthosoma. Thus, the distance between P1 and C1 is considered the prosoma length (PL), and the distance between C1 and PA is considered the opisthosoma length (OL, *dashed arrow*). The distance between P1 and PA denote the idiosoma length (IL, *solid arrow*)

Table 1 Locations of *Stigmaeopsis celarius* (*Sc*) and *S.* sp. (*Ss*) co-occurrence on the same or adjacent shoots

Location	City	Prefecture	Date	Host plant	Taxon ID[a]
(Same shoots)					
Daibutsu Hiking Course	Kamakura	Kanagawa	March 2013	*Pleioblastus chino*	–
Ogijima island	Takamatsu	Kagawa	August 2013	*Pl.* sp.	–
Ryu	Yokonami	Kochi	April 2013	*Pl. argenteostriatus*	–
Gudo	Shimanto	Kochi	September 2014	*Pl. argenteostriatus*	
(Adjacent shoots)					
Hokubu Campus, Kyoto University	Kyoto	Kyoto	January 2008	*Pl. argenteostriatus*	–
Yoshidayama	Kyoto	Kyoto	September 2013	*Pl. argenteostriatus*	–
Kashiwa Campus, Tokyo University	Kashiwa	Chiba	September 2007	*Phyllostachys edulis (Ph. pubescens)*	27, 28, 39
Ohmukai	Kubokawa	Kochi	August 2007	*Pl.* sp.	33, 40
Hataki-cho	Oozu	Ehime	September 2014	*Pl. argenteostriatus*	

Most host plants were *Pleioblastus* spp., except for Moso bamboo in Kashiwa

[a] Taxon ID in Ito and Fukuda (2009)

Crossing experiment

The results of crossing experiments are shown in Table 2. All males of each species successfully entered the nests made by females of the same and other species during the 14 days. Females that mated with males of different species produced significantly fewer eggs than those resulting from intraspecific mating, and the *Sc* pair bore significantly more eggs than the *Ss* pair. The male ratios from intraspecific mating were 0.13 in *Sc* and 0.19 in *Ss*. No females offspring resulted from interspecific mating.

Copulation behavior

The copulation behavior of each cross is summarized in Table 3. The proportions of intraspecific copulation behavior (opisthosoma raising) were not significantly different between *Sc*♀ × *Sc*♂ and *Ss*♀ × *Ss*♂ crosses (Table 3[i]). For *Ss* males, the proportion in the interspecific pair (*Sc*♀ × *Ss*♂) was significantly smaller than in the intraspecific pair (*Ss*♀ × *Ss*♂). In *Sc* males, however, the proportion in the intraspecific pair (*Sc*♀ × *Sc*♂) was not significantly different from the interspecific pair (*Ss*♀ × *Sc*♂). Thus, the response of males to females of other species differed significantly between species.

There was no significant difference in the number of copulation attempts between any combination (Table 3[ii]). However, the period of copulation differed (Table 3[iii]). The copulation periods of *Sc* and *Ss* in intraspecific copulation were not significantly different. The copulation period of *Ss*♀ × *Sc*♂ was 0 (copulation failed) even though *Sc* males tried repeatedly to copulate (Table 3[ii]). In contrast, *Ss* males sometimes copulated with *Sc* females (Table 3[i]). The copulation period of *Sc*♀ × *Ss*♂ did not differ significantly from *Ss*♀ × *Ss*♂, though the average was almost half.

The time from an encounter of a male with a female to the onset of male mounting is shown in Table 3(iv). The time in *Ss*♀ × *Sc*♂ was significantly shorter than in *Sc*♀ × *Sc*♂, indicating that males tended to start copulation sooner in interspecific copulation compared with intraspecific copulation. No significant differences were observed in the other combinations.

Body length

The homogeneity of variances were preliminarily examined for the body length analyses. For IL, the variance between the two species was not significantly different in females ($F_{22,\,23} = 1.385$, $P = 0.44$) but significantly different in males ($F_{25,\,17} = 2.976$, $P = 0.023$).

Table 2 Summary of reciprocal crosses between *Stigmaeopsis celarius* (*Sc*) and *S.* sp. (*Ss*). Proportion of success of male intrusion into the female nest, the number of eggs laid in 14 days, and the sex ratio of offspring (the proportion of males to the total offspring) are presented (Mean ± SD)

Crossing	n	Prop. intrusion	No. eggs[†]	Prop. males[‡]
Sc ♀ × *Sc* ♂	15	1.00 ± 0.00	12.13 ± 5.17^{a}	0.13 ± 0.09^{a}
Sc ♀ × *Ss* ♂	18	1.00 ± 0.00	1.89 ± 1.18^{b}	1.00 ± 0.00^{b}
Ss ♀ × *Sc* ♂	16	1.00 ± 0.00	1.88 ± 1.50^{b}	1.00 ± 0.00^{b}
Ss ♀ × *Ss* ♂	17	1.00 ± 0.00	9.18 ± 1.13^{c}	0.19 ± 0.10^{a}

Significant differences within a column are indicated by different letters

[†] Tukey's multiple comparison test

[‡] Ryan's procedure (both $P < 0.05$)

Table 3 Parameters of copulatory behavior in reciprocal crosses between *Stigmaeopsis celarius* (*Sc*) and *S.* sp. (*Ss*)

Cross	(i) Prop. copulation[†]		(ii) No. of copulation attempts[‡]		(iii) Copulation period (s)[‡]		(iv) Time for mounting (s)[‡]	
	n	%	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD
Sc♀ × *Sc*♂	13	0.62^{ab}	13	0.6 ± 0.5a	8	89.5 ± 48.6a	8	4,829 ± 4,370a
Sc♀ × *Ss*♂	13	0.23^{a}	13	1.4 ± 3.2a	3	45.7 ± 16.5a	3	2,688 ± 1,065ab
Ss♀ × *Sc*♂	15	0.67^{ab}	15	10.4 ± 13.2a	10	0.0 ± 0.0b	10	929 ± 1,825b
Ss♀ × *Ss*♂	14	0.86^{b}	14	1.4 ± 1.1a	12	80.5 ± 76.5a	12	5,253 ± 4,486a

n Indicates the number of males in each cross, (i) proportion of males showing copulatory behavior (opisthosoma raising), (ii) the number of copulation attempts by males, (iii) copulation period of copulating males, and (iv) time required for pre-copulatory mounting from first contact with a female

Significant differences within a column are indicated by different letters

[†] Tukey's multiple comparison test for proportion data

[‡] Steel-Dwass test (both $P < 0.05$)

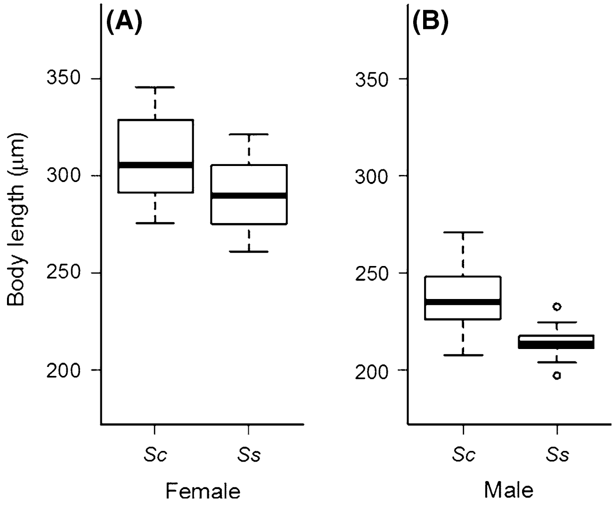

Fig. 3 Body length measured as the distance between the bases of setae P1 and PA (see Fig. 2, IL). Unit: µm. **a** Females and **b** males. *Boxes* denote the interquartile ranges (IQR) between the first and third quartiles (25th and 75th percentiles, respectively), and the thick line inside denotes the median. *Whiskers* represent the lowest and highest values within 1.5 IQL from the first and third quartiles, respectively. Values outside these ranges are separately shown by *open circles*. Body length differed significantly between the sexes ($P < 0.05$, t test)

The variance between the two species for the male OL was significantly different ($F_{24,\ 17} = 3.279$, $P = 0.014$). The variance in the male PL was also significantly different between species ($F_{24,\ 17} = 5.421$, $P < 0.001$). Thus, male body lengths were analyzed using the Welch t test assuming unequal variance. Female size was analyzed by the ordinary t test.

The IL of *Sc* was significantly longer than that of *Ss* in both sexes (Fig. 3, female *Sc*: 308.3 ± 22.6 µm, female *Ss*: 291.2 ± 19.3 µm, $t_{45} = 2.791$, $P < 0.008$; male *Sc*: 235.0 ± 14.3 µm, male *Ss* 214.4 ± 8.3 µm, $t_{40.965} = 6.063$, $P < 0.001$). The male OL of *Sc* was significantly longer compared with *Ss* (*Sc*: 148.4 ± 10.7 µm, *Ss*: 130.6 ± 5.9 µm, $t_{38.808} = 6.969$, $P < 0.001$), whereas the male PL did not differ significantly between species (*Sc*: 87.1 ± 10.0 µm, *Ss*: 83.8 ± 4.3 µm, $t_{34.662} = 1.446$, $P = 0.16$). Therefore, interspecific differences in IL were attributed to the variation in the OL.

The IL of females, males, and the difference between them was not significantly correlated with any of the three copulatory behaviors in either cross ($P > 0.05$) except for the time for mounting (iv) in *Ss*♀ × *Sc*♂ ($r_8 = -0.64$, $n = 10$, $P < 0.05$). The correlations were also not significant when the data from all the crosses were pooled ($n = 55$ for copulation data, $n = 33$ for mounting data). Furthermore, the correlation between the OL and each copulation behavior was not significant for any cross ($P > 0.05$).

Discussion

Our field survey suggests that *Sc* and *Ss* inhabit the same shoots of Nezasa bamboo (*Pleioblastus* spp.) in various areas of southwestern Japan. Thus, individuals of these species may encounter one another in these areas. Interspecific mating at the boundary area has been reported in various spider mites, and male offspring are over-produced and sterile F1 females partly emerge in the cross of two forms of *S. miscanthi* (Sato et al. 2000b). In either case, the fitness of the parents should be decreased by a loss of reproductive opportunity to produce offspring with an appropriate sex ratio. Thus, the mechanisms to impede interspecific mating would be evolved in such contact zones.

The crossing experiments suggest that *Sc* and *Ss* are reproductively isolated. In the interspecific crosses, all developed offspring were males, suggesting that fertilization did not occur, though short-time copulation was observed only in a few pairs. Notably, significantly fewer eggs were produced in the interspecific crosses compared with intraspecific crossing. Because copulation itself, either with the same or different species, strongly increases the fecundity of *Stigmaeopsis* species (Saito 1987; Sato et al. 2000a, b), the decrease in egg numbers suggests that copulation itself was not successful in the interspecific crosses. Therefore, precopulatory reproductive isolation is the major factor inhibiting gene flow between these species. In contrast, because the males of each species could intrude the nests of the other species, the effects of the web nests to prevent the intrusion of males of different species (Mori 2000) were negligible. Thus, on the leaves or shoots that both species inhabit, males can move around the nests of both species and may encounter the females of the same or different species.

Our study revealed asymmetrical copulation behavior of the two species in interspecific crosses. In *Sc*♀ × *Ss*♂, *Ss* males made little attempt to copulate with *Sc* females. The contact of genitalia (apparent copulation) only occurred in a few pairs, but we observed no significant depression in hatchability from the death of hybrid eggs as in *S. miscanthi* (Sato et al. 2000b) and no female progeny (evidence of fertilization) in any pair. Therefore, we conclude that sperm transfer was unsuccessful in this cross. At present, the factors that deter *Ss* males from copulating with *Sc* females are unclear, but pheromone differences between the species might be a plausible explanation because males of various mite species use female pheromones for mate recognition (Cone 1985; Shimizu et al. 2001).

In contrast, in *Ss*♀ × *Sc*♂ crosses, males approached females as often as conspecific females, but they never succeeded in copulation and could not fertilize eggs. This could be attributed to the difference in body length between the two species. Though the lengths of male prosoma were similar in the two species, male opisthosoma, and thus idiosoma, of *Sc* was much longer than that of *Ss*. During the experiments, we repeatedly observed that the opisthosoma of *Sc* was too long to appropriately insert the aedeagus into the genitalia of a *Ss* female body. This suggests that the mismatch in body size between the sexes affects reproductive isolation, and the long opisthosoma of *Sc* functions as a barrier to interspecific mating. Such effects of mismatches on copulation success are reported in millipidae (Tanabe and Sota 2008) and carabid beetles (Okuzaki et al. 2010), in which sexual differences in body size play an important role in reproductive isolation. In sticklebacks with external fertilization, the probability of interspecific mating is strongly correlated with body size (Schluter and Nagel 1995; Nagel and Schluter 1998; Schluter 1998). In the present study, however, relationships between body size and copulatory behavior were not significant, presumably because of small sample sizes. Further studies are needed to elucidate the effect of size difference on copulation success in spider mites.

The results from the *Ss*♀ × *Sc*♂ cross may be related to reproductive interference (Gröning and Hochkirch 2008). Interspecific hybridization leads to exclusion without resource competition, local extinction, and causes parapatry (Bull 1991; Kuno 1992). Several studies have reported the occurrence of interspecific hybridization in spider mites, such as *Tetranychus* (Ben-David et al. 2009) and *Panonychus* (Fujimoto et al. 1996;

Takafuji et al. 1996). Recent studies on insects suggest that even without interspecific copulation, female fitness is decreased only by the harassment behavior of males of different species, eventually leading to the extinction of one species (Hochkirch et al. 2007; Kishi et al. 2009; Kyogoku and Nishida 2012, 2013). In the present case, even if *Sc* males could not copulate with *Ss* females, copulatory attempts were repeated many times, and, thus, fitness loss by *Sc* male harassment is possible in the contact zone. We hypothesize that if the body sizes of the mates of two species match, females suffer from fitness loss by producing excessive male offspring or sterile hybrids (Sato et al. 2000b), otherwise females incur harassment by males of different species. To test this hypothesis, we should estimate the fertilization status of interspecies crossing as well as the magnitude of harassment imposed by males of different species living in the same habitat.

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